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The Impact of Differential Expression of Extracellular Matrix Metalloproteinase Inducer, Matrix Metalloproteinase-2, Tissue Inhibitor of Matrix Metalloproteinase-2 and PDGF-AA on the Chronicity of Venous Leg Ulcers

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Introduction. *Alteration in the expression of extracellular matrix metalloproteinase inducer (EMMPRIN), matrix metalloproteinase-2 (MMP-2), tissue inhibitors of matrix metalloproteinases (TIMP-2) and platelet derived growth factor (PDGF-AA) may contribute to poor healing in venous leg ulcers.*
Aim. *The aim of this study is to determine the expression of EMMPRIN, MMP-2, TIMP-2 and PDGF-AA in the ulcer exudates and perivascular tissue of healing and non-healing chronic venous ulcers.*
Patients, materials and methods. *Forty patients with chronic venous ulcers were included in this study, with a mean age of 60 years. Eleven patients were males and 29 were females. All patients had normal ankle brachial index and a venous ulcer of at least 8 weeks duration.*
Immuno-histochemistry using monoclonal antibodies to PDGF-AA, MMP-2, TIMP-2 and EMMPRIN was carried out on paraffin embedded punch biopsy skin specimens from the ulcer edge. Enzyme linked immunosorbent assay for PDGF, MMP-2 and TIMP-2 were carried out on wound fluids collected from patients. The ulcer size and character at the initial assessment and after 8 weeks were assessed to determine the status of ulcer healing.
Results. *No significant difference was seen in the expression of TIMP-2, MMP-2 and EMMPRIN between the two groups. However, in the non-healing group high levels of MMP-2 and low levels of TIMP-2 in the wound fluid suggest a strong correlation of these two markers in the state of healing. Analysis of wound fluid by ELISA demonstrated high PDGF-AA in the healing group* ($p = 0.021$). *Significantly increased levels of PDGF-AA* ($p < 0001$) *was noted in the perivascular area on immuno-histochemistry of healing ulcers. These data suggest that PDGF-AA plays an important role in healing of venous ulcers.*
Conclusion. *Non-healing venous ulcers are associated with greater activity MMP-2 activity. The ratio of MMPs to their inhibitors TIMPs, dictate the rate of healing of the ulcers. PDGF-AA activity is associated with ulcer healing, though the mechanism is unclear. EMMPRIN expression in chronic venous ulcers probably parallels the chronicity of the condition rather than propagate it. However, further studies with larger samples are needed.*

Keywords: Matrix metalloproteinase inducer; Matrix metalloproteinase-2; Tissue inhibitors of matrix metalloproteinases; Platelet derived growth factor; Venous ulcer.

Introduction

Chronic venous leg ulcer is common in the aging population. Venous disease accounts for 81% of ulcers[1] and the prevalence of leg ulcers is 1.03% in patients aged over 70 years. Ambulatory venous hypertension is caused by superficial and deep venous incompetence and alteration of the skin microcirculation eventually leads to ulceration. Successful wound healing depends on a complex interplay of cytokines and growth factors involving inflammation, cell proliferation, extracellular matrix deposition and remodelling.[2,3] Matrix metalloproteinases (MMP's) are secreted by various cells types (fibroblasts, leukocytes, keratinocytes) as proenzymes and are activated by membrane type MMP-s or by serine proteases. MMP's have been implicated with excessive

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extracellular matrix degradation in chronic venous ulcers with the resultant failure of completion of the healing process. Enhanced proteolytic activity attributable to MMP's has been demonstrated in venous ulcers.[4]

Aim

The study was designed to determine the expression of, EMMPRIN, MMP-2, TIMP-2 and PDGF-AA in the perivascular and stromal areas of healing and non-healing venous ulcers. Our aim is to assess the differential expression of the above factors and alteration in their pattern in relation to the rate of healing in a group of patients with venous ulcers.

Patients, Materials and Methods

Forty patients attending the venous unit were recruited into the study. In our department patients are managed following clinical and duplex ultrasound examination to assess the patency and competence of deep and superficial veins. Patients were included with age greater than 50 years (mean age 60 years), venous leg ulcer duration of at least 8 weeks and ankle brachial index greater than 0.9. All patients included in this study were of CEAP clinical stage 6. Exclusion criteria included deep venous reflux or history of deep venous thrombosis, cigarette smoking, history of chronic renal or liver disease, rheumatoid arthritis or diabetes and usage of steroids or immuno-suppression. All patients were treated with Profore® (Smith & Nephew, UK) graduated compression bandaging for 8 weeks. Ethics approval was obtained from local authority ethics committee, Western Health Board and all patients gave their informed consent to inclusion in this study.

The ulcer size and slough at ulcer base was recorded at the first visit and the healing state was assessed at subsequent visit 8 weeks later in relation to change in size, slough, wound contraction and presence of granulation tissue. Wounds were regarded as healing if there was a decrease in the size of the ulcer ($>20\%$ decrease in surface area), decrease in slough and development of healthy granulation tissue. Patients were allocated into healing and non healing groups according to the above criteria.

Specimen collection was carried out at the first visit. Wound fluid exudates were collected from the non adherent dressing covering the ulcer using sterile micro-pipettes and frozen immediately at $-80\,^{\circ}C$. The collection was done on wounds, which had been dressed not longer than 36 h prior to clinic attendance. This was followed by 4 mm punch biopsy of the ulcer bed adjacent to the ulcer edge under local anaesthetic and immediate fixation in formalin.

Immunoassay was carried out on the wound fluid that was thawed and assayed at room temperature as per the ELISA (R&D) protocol for PDGF-AA, MMP-2 and TIMP-2. The results were calculated after averaging the duplicate readings for each standard, control and the sample. The optical density was plotted to give the best-fit curve. A standard curve was generated for each set of samples assayed. The units of the results were recorded in nanograms for TIMP-2 and MMP-2 and picograms for PDGF-AA after correction for the dilution factor.

Immuno-histochemistry was carried out on paraffin embedded skin biopsy sections taken at the edge of the ulcer. Sections were initially retrieved from paraffin, followed by heat induced antigen retrieval with EDTA and subsequently incubated with monoclonal antibodies to EMMPRIN, MMP-2, PDGF-AA and TIMP-2. The final step was counterstaining and dehydration of the sections according to the manufacturer's instructions. The histological sections were then graded semi-quantitatively into negative and positive by the intensity of staining in the stromal and perivascular zones, by two pathologists blinded to patient groups.

Statistical analysis was carried out using SPSS software (SPSS Inc., Chicago, IL, USA). The independent samples t test was used for quantitative continuous data while the chi-square test was used for nominal data arising from immuno-histochemistry scores. In both tests, a p value of <0.05 was considered significant. The descriptors used in the text and figure and tables are the mean and standard deviation.

Results

Of the 40 patients that were recruited for study 11 were male and 29 female. Twenty ulcers were improving and 20 had no change or deteriorated.

Immunoassay (ELISA) was performed for TIMP-2, MMP-2 and PDGF-AA on the wound fluid and the mean immunoassay for TIMP-2 in the healing group was 1.36 SD 0.96 ng/ml and 0.83 SD 0.77 ng/ml in the non-healing group. Assay of MMP-2 showed the mean values of 117 SD 61 ng/ml and 133 SD 12 ng/ml in the healing and non-healing groups, respectively. There was no statistical significance between the two groups for both TIMP-2 and MMP-2 but a trend was noticed that higher levels were seen in non-healing ulcers (Table 1). Interestingly a strong correlation of TIMP-2 and MMP-2 levels was recorded on ELISA analysis in

Table 1. Results of ELISA analysis of wound fluid for MMP-2 and TIMP-2

Mean concentration (ng/ml)	MMP-2	TIMP-2
Healing ulcer	117 (SD 61)	1.36 (SD 0.96)
Non healing ulcer	133 (SD 12)	0.83 (SD 0.77)

Levels of MMP-2 and TIMP-2 in wound fluid. No statistically significant association with wound healing was found.

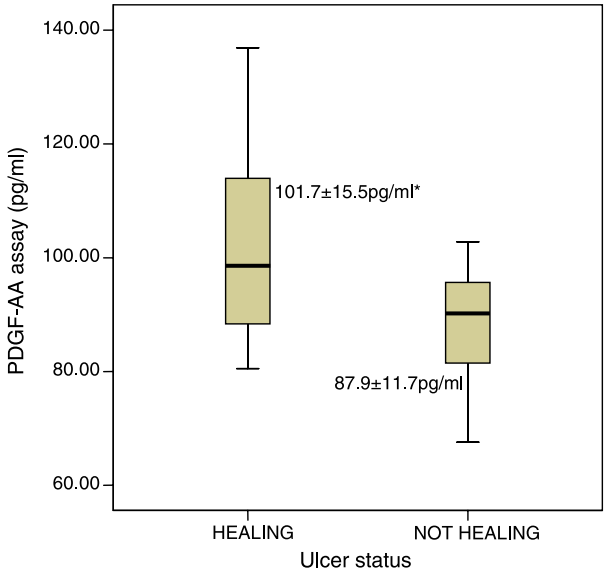

Fig. 1. Box plot showing the Immunoassay for PDGF-AA levels. Box plot shows median, inter-quartile range and 1.5 times inter-quartile range. Higher values were observed in the healing group ($p < 0.021$, *t*-test).

the non-healing group (with the r value = 0.94 and the p value = 0.0001), where the TIMP-2 levels were lower; there was greater activation of MMP-2.

On immunoassay of the wound fluid PDGF-AA was present at concentration of 102 SD 16 pg/ml in the healing group in comparison to 88 SD 12 pg/ml in the non-healing group of ulcers This difference was statistically significant. ($p < 0.021$), (Box plot Fig. 1).

On immuno-histochemistry, elevated staining pattern of PDGF-AA was noted in the healing group of patients compared to the non-healing group. This was reflected in the significant difference in the expression of PDGF-AA between the two groups ($p < 0.0001$).

Immuno-histochemical staining of TIMP-2 was negative in both groups of patients. The TIMP-2 levels were probably too low in the tissues to show any discernable staining pattern. Perivascular or stromal staining for expression of MMP-2 by immuno-histochemistry was successful but did not show a significant difference between the two groups. Staining for EMMPRI N did not show significant difference between the healing and non-healing groups of patients (Table 2).

Discussion

There is no uniform consensus on the exact micro-circulatory changes in the pathogenesis of venous ulcers but it has been postulated that venous hypertension results in perivascular leakage of fibrinogen that polymerizes to form perivascular fibrin cuff which impairs the diffusion of oxygen and nutrients and thus, lead to ulcer formation.[5] Various studies tried to explain the molecular biology of chronic venous ulcers such as the fibrin cuff theory,[5] the trap hypothesis[6] and the white cell trapping theory[7] and none of these theories completely explains the mechanism and the chronicity of ulceration and research into this area is still quite extensive.

Extracellular matrix metalloproteinase inducer (EMMPRIN) is a membrane bound protein that has been identified in normal keratinocytes.[8] It causes denovo synthesis of MMP-1, MMP-2 and MMP-3 when exposed to human fibroblasts[9] and it may promote the expression of MMP's in chronic venous ulcers. Activation of MMP-2 has been immuno-localized in the dermal-epidermal junction as well as in perivascular areas in lipodermatosclerosis.[10] EMMPRIN enhances turnover of extracellular matrix and unrestrained MMP activity, as seen by the elevated expression of EMMPRIN and MMP-2 in the perivascular regions in venous leg ulcers and the intense expression of EMMPRIN, MMP-2, MT1-MMP and MT2-MMP in dermal structures of venous leg ulcers.[4] Our results in the two groups of patients by immuno-histochemistry showed no significant difference in

Table 2. Immuno-histochemistry analysis summary

	EMMPRIN		MMP-2		PDGF-AA	
	Positive	Negative	Positive	Negative	Positive	Negative
Healing ulcer	9	9	11	8	17	1
Non healing ulcer	11	9	17	3	2	18

Two samples inadequate for EMMPRIN and one sample was inadequate for PDGF-AA and MMP-2. Summary expressions of EMMPRIN, MMP-2 and PDGF-AA analysis by immuno-histochemistry.

the staining patterns of EMMPRIN and it is probable that expression of this membrane bound protein is seen in chronic ulcers irrespective of the healing state of the ulcer. This supports the finding by Herouy *et al.*,[4] that its expression is associated with enhanced matrix turnover that is a feature in chronic ulcers.

Platelet-derived growth factor (PDGF) contained in human platelets in its AA isoform is present at sites of injury during the acute phase of the wound repair response[11] and impaired healing states may be associated with reduced expression of this factor.

PDGF stimulates the secretion of other growth factors involved in the healing process and the production of matrix components such as collagen[12] and hyaluronic acid.[13] At late stages of wound healing it promotes the contraction of collagen matrices *in vitro*[14] and stimulates the secretion of collagenase.[15] Reduction in the expression of PDGF-AA type receptor expression has been shown in wounded skin[16] of animal models, suggesting that healing impaired states may be associated with reduced expression of this factor. Pierce *et al.*[17] showed up-regulation of PDGF-AA in capillaries and fibroblasts of acute and chronic wounds treated with PDGF-BB but not in non-healing dermal ulcers or normal skin. Similarly, we found increased levels of PDGF-AA expression in perivascular areas of healing venous ulcers and not chronic non-healing wounds. PDGF plays an important role in wound healing by stimulating smooth cell migration and angiogenesis[18].

The injurious effect of matrix metalloproteinases and their probable role in the aetiology of venous ulcer is seen by the increased expression of the messenger RNA and protein of MMP-2 and TIMP-2 in patients with lipodermatosclerosis compared to normal controls.[10] Their proteolytic activity in the acute and chronic wound environment has been shown by the elevated activity of MMP-9 and to a lesser extent of MMP-2 activity. In addition the levels of matrix metalloproteinases activity in wound fluid samples collected in patients with healing venous ulcers were shown to decrease significantly as healing progressed.[19] Our results did not show any significant difference in the levels of MMP-2 in the two groups of patients but the mean level of MMP-2 expression was higher in the non healing group at 133 SD 12 ng/ml compared to 117 SD 61 ng/ml in the healing group. As we only looked at MMP-2 and not other matrix metalloproteinases it is probable that other matrix metalloproteinases as described by Woessner *et al.*,[20] may be responsible for the degradation and enhanced matrix turnover in these patients wounds.

The tissue inhibitors of matrix metalloproteinases (TIMPs) regulate the proteolytic activity of MMPs by inhibition of activated MMP in a 1:1 non-covalent binding[21] and in normal healing wounds both play an important role in the remodelling of the wound bed. Lack of TIMP-2 near the migrating epithelial wound edges might contribute to uncontrolled activity of MMP-2 in chronic ulcers.[22] When an imbalance primarily exists between levels of matrix metalloproteinases and their inhibitors, MMPs bind their TIMPs to attenuate their capacity to regulate the proteinase activity in tissue. Such MMP/TIMP complex is seen in abundance[23] in the wound fluid of pressure ulcers. Further confirmation of this is seen by the increased levels activated enzyme and pro-enzyme of MMP-2 in chronic leg ulcers.[24] In our study, the mean level of TIMP-2 was higher in the healing group than in the non-healing group as was demonstrated by immunoassay. In the non- healing group of patients we noted a correlation between higher MMP-2 and lower TIMP-2 levels ($p < 0.0001$), suggesting that non-healing ulcers have a higher levels of proteinase activity. This effect was observed in other studies regarding aberrant wound healing and healing of pressure ulcers.[21,22]

Conclusion

PDGF-AA activity is associated with ulcer healing, though the mechanism is unclear. EMMPRIN expression in chronic venous ulcers probably parallels the chronicity of the condition rather than propagating it. However, further studies with larger samples are needed.

The healing of venous ulcers is dependent on the activity of MMPs. Non-healing venous ulcers are associated with greater activity MMP-2. The ratio of MMPs to their inhibitors TIMPs, dictate the rate of healing of the ulcers. Whether the ratio of TIMP/MMP in the wound fluid could indicate the future response of these ulcers to treatment is a subject of further study.

References

1 O'Brien JF, Grace PA, Perry IJ, Burke PE. Prevalence and aetiology of leg ulcers in Ireland. *Ir J Med Sci* 2000;**169**(2):110–112.
2 Hackam DJ, Ford HR. Cellular, biochemical, and clinical aspects of wound healing. *Surg Infect (Larchmt)* 2002;**3**(Suppl 1):S23–S35.
3 Hunt TK, Hopf H, Hussain Z. Physiology of wound healing. *Adv Skin Wound Care* 2000;**13**(Suppl 2):6–11 [Review].
4 Norgauer J, Hildenbrand T, Idzko M, Panther E, Bandemir E, Hartmann M *et al*. Elevated expression of extracellular matrix metalloproteinase inducer (CD147) and membrane-type matrix metalloproteinases in venous leg ulcers. *Br J Dermatol* 2002; **147**(6):1180–1186.

5 Burnand KG, Whimster I, Naidoo A, Browse NL. Pericapillary fibrin in the ulcer-bearing skin of the leg: the cause of lipodermatosclerosis and venous ulceration. *Br Med J (Clin Res Ed)* 1982;**285**(6348):1071–1072.
6 Falanga V, Eaglstein WH. The 'trap' hypothesis of venous ulceration. *Lancet* 1993;**341**(8851):1006–1008.
7 Coleridge Smith PD, Thomas P, Scurr JH, Dormandy JA. Causes of venous ulceration: a new hypothesis. *Br Med J (Clin Res Ed)* 1988;**296**(6638):1726–1727.
8 DeCastro R, Zhang Y, Guo H, Kataoka H, Gordon MK, Toole B *et al*. Human keratinocytes express EMMPRIN, an extracellular matrix metalloproteinase inducer. *J Invest Dermatol* 1996;**106**(6):1260–1265.
9 Guo H, Zucker S, Gordon MK, Toole BP, Biswas C. Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. *J Biol Chem* 1997;**272**(1):24–27.
10 Herouy Y, May AE, Pornschlegel G, Stetter C, Grenz H, Preissner KT *et al*. Lipodermatosclerosis is characterised by elevated expression and activation of matrix metalloproteinases: implications for venous ulcer formation. *J Invest Dermatol* 1998; **111**(5):822–827.
11 Soma Y, Dvonch V, Grotendorst GR. Platelet-derived growth factor AA homodimer is the predominant isoform in human platelets and acute human wound fluid. *FASEB J* 1992;**6**(11):2996–3001.
12 Canalis E, Lian JB. Effects of bone associated growth factors on DNA, collagen and osteocalcin synthesis in cultured fetal rat calvariae. *Bone* 1988;**9**(4):243–246.
13 Heldin P, Pertoft H, Nordlinder H, Heldin CH, Laurent TC. Differential expression of platelet-derived growth factor alpha- and beta- receptors on fat-storing cells and endothelial cells of rat liver. *Exp Cell Res* 1991;**193**(2):364–369.
14 Zagai U, Fredriksson K, Rennard SI, Lundahk J, Skold CM. Platelets stimulate fibroblast-mediated contraction of collagen gels. *Respir Res* 2003;**4**(1):13.
15 Bauer EA, Cooper TW, Huang JS, Altman J, Deuel TF. Stimulation of *in vitro* human skin collagenase expression by platelet-derived growth factor. *Proc Natl Acad Sci U S A* 1985; **82**(12):4132–4136.
16 Beer HD, Longaker MT, Werner S. Reduced expression of PDGF and PDGF receptors during impaired wound healing. *J Invest Dermatol* 1997;**109**(2):132–138.
17 Pierce GF, Tarpley JE, Tseng J, Bready J, Chang D, Kenney WC *et al*. Detection of platelet-derived growth factor (PDGF)-AA in actively healing human wounds treated with recombinant PDGF-BB and absence of PDGF in chronic nonhealing wounds. *J Clin Invest* 1995;**96**(3):1336–1350.
18 Risau W, Drexler H, Mironov V, Smits A, Siegbahn A, Funa K *et al*. Platelet-derived growth factor is angiogenic *in vivo*. *Growth Factors* 1992;**7**(4):261–266.
19 Trengove NJ, Stacey MC, MacAuley S, Bennett N, Gibson J, Burslem F *et al*. Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. *Wound Repair Regen* 1999;**7**(6):442–452.
20 Woessner Jr JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 1991;**5**(8): 2145–2154.
21 Mcintush EW, Smith MF. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in ovarian function. *Rev Reprod* 1998;**3**(1):23–30.
22 Vaalamo M, Leivo T, Saarailho-Kere U. Differential expression of tissue inhibitors of metalloproteinases (TIMP-1,-2,-3 and -4) in normal and aberrant wound healing. *Hum Pathol* 1999;**30**(7):795–802.
23 Yager DR, Zhang LY, Liang HX, Diegelmann RF, Cohen IK. Wound fluids from human pressure ulcers contain elevated matrix metalloproteinase levels and activity compared to surgical wound fluids. *J Invest Dermatol* 1996; **107**(5):743–748.
24 Wysocki AB, Staiano-Coico L, Grinnell F. Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. *J Invest Dermatol* 1993;**101**(1):64–68.

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